AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

OsaR (PA0056) Functions as a Repressor of the Gene *fleQ* Encoding an Important Motility Regulator in *Pseudomonas aeruginosa*

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ABSTRACT FleQ plays a crucial role in motility and biofilm formation by regulating flagellar and exopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. It has been reported that the expression of FleQ is transcriptionally downregulated by the virulence factor regulator Vfr. Here, we demonstrated that a LysR-type transcriptional regulator, OsaR, is also capable of binding to the promoter region of *fleQ* and repressing its transcription. Through gel shift and DNase I footprinting assays, the OsaR binding site was identified and characterized as a dual LysR-type transcriptional regulator box (AT-N_{11}-AT-N_7-A-N_{11}-T). Mutation of the A-T palindromic base pairs in the *fleQ* promoter not only reduced the binding affinity of OsaR *in vitro* but also derepressed *fleQ* transcription *in vivo*. The OsaR binding site was found to cover the Vfr binding site; knockout of *osaR* or *vfr* separately exhibited no effect on the transcriptional level of *fleQ*; however, *fleQ* expression was repressed by overexpression of *osaR* or *vfr*. Furthermore, simultaneously deleting both *osaR* and *vfr* resulted in an upregulation of *fleQ*, but it could be complemented by the expression of either of the two repressors. In summary, our work revealed that OsaR and Vfr function as two transcriptional repressors of *fleQ* that bind to the same region of *fleQ* but work separately.

IMPORTANCE *Pseudomonas aeruginosa* is a widespread human pathogen, which accounts for serious infections in the hospital, especially for lung infection in cystic fibrosis and chronic obstructive pulmonary disease patients. *P. aeruginosa* infection is closely associated with its motility and biofilm formation, which are both under the regulation of the important transcription factor FleQ. However, the upstream regulatory mechanisms of *fleQ* have not been fully elucidated. Therefore, our research identifying a novel regulator of *fleQ* as well as new regulatory mechanisms controlling its expression will be significant for better understanding the intricate gene regulatory mechanisms related to *P. aeruginosa* virulence and infection.

KEYWORDS *Pseudomonas aeruginosa*, transcriptional regulation, OsaR, *fleQ*

Pseudomonas aeruginosa is a ubiquitous Gram-negative pathogenic bacterium that causes acute and chronic infections in immunocompromised individuals, such as burn victims and cystic fibrosis patients (1–3). *P. aeruginosa* pathogenesis is determined by its production of various virulence factors, for example, flagella, motility, and biofilm formation (4). The expression of these traits is under complex gene regulation, which involves numerous transcriptional regulators, regulatory RNAs, and σ factors (5).

The NtrC family transcription factor FleQ is a major regulator in the process of flagellar biosynthesis (6), as it works together with the alternate sigma factor σ^{54} (RpoN) to activate the transcription of most flagellar genes (7–9). The *fleQ* mutant exhibits loss of motility and the ability to synthesize flagellin or assemble a flagellum (7).

FleQ activity is posttranscriptionally downregulated by its antiactivator FleN, which binds to FleQ to inhibit FleQ ATPase activity (10, 11). Sigma factor σ^{70} is involved in the activation

Citation Ma Y, Liu Y, Bi Y, Han X, Jin Y, Xu H, Qiao M. 2021. OsaR (PA0056) functions as a repressor of the gene *fleQ* encoding an important motility regulator in *Pseudomonas aeruginosa*. J Bacteriol 203:e00145-21. https://doi.org/10.1128/JB.00145-21.

Editor George O'Toole, Geisel School of Medicine at Dartmouth

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Received 11 March 2021
Accepted 22 July 2021
Accepted manuscript posted online 2 August 2021
Published 23 September 2021

of *fleQ* transcription (12). AmrZ and Vfr have been reported as two transcriptional repressors of *fleQ*. The alginate and motility regulator AmrZ functions globally in the regulation of various genes, such as the *psl* operon, involved in Psl exopolysaccharide synthesis (13), and *algD*, involved in alginate production (14). *fleQ* promoter activity was approximately 4-fold higher in an *amrZ* mutant (15), while transcriptome sequencing (RNA-seq) analysis indicated that *fleQ* transcription was repressed 8-fold by AmrZ (16). The virulence regulator Vfr is another global transcriptional regulator of a number of genes, such as *toxA* and *recA*, involved in exotoxin A production, *lasR* and *rhlR*, involved in quorum sensing, and *exsA*, involved in the type III secretion system (17–19). Overexpression of Vfr downregulates *fleQ*, but, peculiarly, *fleQ* promoter activity was not upregulated in the *vfr* mutant compared with the wild-type (WT) strain (12).

OsaR was previously identified by our group as a transcription factor belonging to the family of LysR-type transcriptional regulators (LTTRs) (20). A number of LTTRs function as global regulators of the genes involved in metabolism, cell division, quorum sensing, virulence, motility, nitrogen fixation, oxidative stress responses, toxin production, attachment, and secretion (21). We performed chromatin immunoprecipitation sequencing (ChIP-seq) to identify genes affected directly by OsaR; the bioinformatic analysis yielded many peaks, and genes were selected by assignment to these peaks. *fleQ* was one of the selected genes that was putatively regulated by OsaR. In this report, we confirmed the interaction between OsaR and *fleQ* and ultimately identified a novel regulatory mechanism involving two transcriptional repressors, OsaR and Vfr, that coregulate the expression of *fleQ*.

RESULTS

OsaR binds to the intergenic region upstream of *fleQ in vivo* and *in vitro*. ChIP-seq was conducted to explore the putative interaction of OsaR and its target operons or genes (Seqhealth, China). A set of gene loci was preliminarily identified by peak calling, and those with fold enrichment above 2.0 were selected for further verification (see the supplemental material). Data analyses indicated two peak regions that may contain an OsaR binding site that were relevant to the gene *fleQ* and the *fleSR* operon, respectively (Fig. 1A). *fleQ* and *fleSR* are both crucial in the biosynthesis of flagella, and their locations in the *P. aeruginosa* genome are adjacent (6, 7, 22). Therefore, we performed gel shift assays to validate the binding of OsaR to the two regions. DNA fragments representing the whole intergenic region upstream of *fleQ* were mixed with OsaR protein, followed by polyacrylamide gel electrophoresis. Considering that the center of the *fleSR* peak region was actually within the coding sequence of the *fleS* gene, as shown in Fig. 1A, 4 overlapping DNA fragments (*fleS1*, *fleS2*, *fleS3*, and *fleS4*) covering both the intergenic region and the coding region were assayed. As shown in Fig. 1B, OsaR caused a modest motility shift of the *fleQ* intergenic fragment, while no shifted band was observed for *fleSR*, probably due to nonspecific binding that causes false positives in the ChIP-seq. Together, the ChIP-seq data and the gel shift assay results demonstrated that OsaR is capable of binding to the intergenic region upstream of *fleQ* both *in vivo* and *in vitro*.

***osaR* overexpression downregulates *fleQ* and swimming motility.** Our previous study found that OsaR, as a typical LTTR, autoregulates itself positively (20). To test whether OsaR regulates *fleQ* transcription and, if it does, whether the regulation is positive or negative, we detected the mRNA levels and promoter activities of *fleQ* in a WT strain (PAK19), *osaR* mutant (ΔosaR19), and *osaR* overexpressing strain (osaROE). By reverse transcription-quantitative PCR (RT-qPCR), we found that *fleQ* mRNA level was not affected by *osaR* knockout but significantly decreased when *osaR* was overexpressed (Fig. 2A). This result was consistent with that of the reporter assay. We constructed a reporter plasmid, P_{fleQ}-*lacZ*, by fusing the *fleQ* promoter region to a promoterless *lacZ* gene. We then transformed the reporter plasmid into PAK19, ΔosaR19, and osaROE strains. The β-galactosidase assays suggested that *fleQ* promoter activity was not significantly different in the *osaR* mutant but obviously reduced in the *osaR*-overexpressing strain compared with that in the WT strain (Fig. 2B). Since *fleQ* is essential to

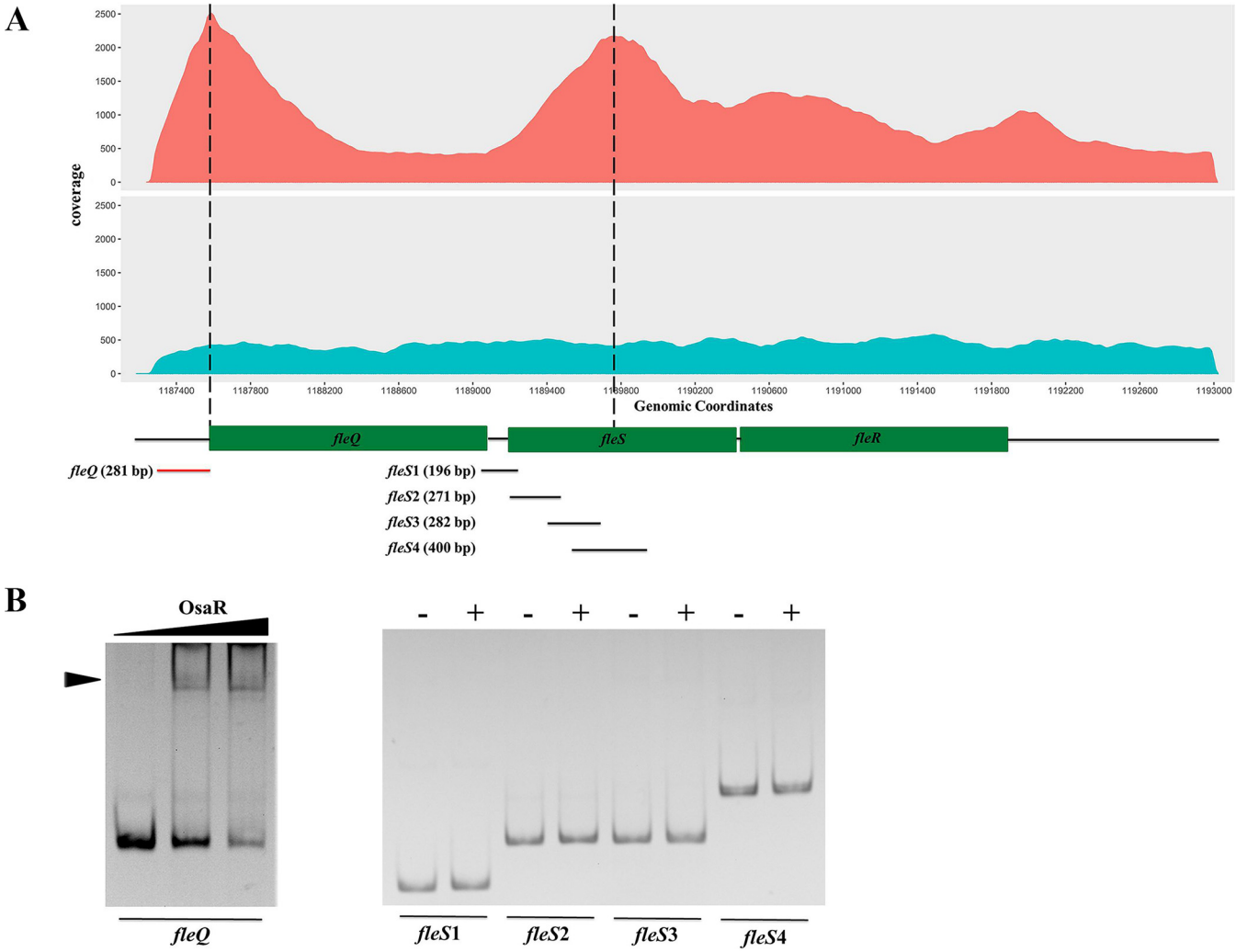

FIG 1 OsaR binds to the intergenic region upstream of *fleQ in vivo* and *in vitro*. (A) OsaR immunoprecipitation reads (in red) were plotted against the number of reads from the nonimmunoprecipitated DNA (in blue). The ordinate indicates the coverage depth of the reads at a certain genomic location. The region represented by the abscissa corresponds to the genomic location from coordinates 1187000 to 1193000. The locations of the *fleQ* and *fleSR* genes are represented in boxes below the abscissa. Peaks (reads, >1,500) represented by DNA accumulations are marked with dashed lines. (B) Gel shift assays of OsaR and the intergenic DNA upstream of *fleQ* or the DNA fragments covering both the intergenic region and coding region of *fleS* (*fleS1* to *fleS4*). The protein-DNA complex is indicated by an arrowhead. The length and location of the fragments are shown in panel A; red and black colors indicate shift or no shift, respectively. Purified OsaR was used in a concentration gradient of 0, 20 nM, and 40 nM for *fleQ* or 0 (−) and 40 nM (+) for *fleS1* to *fleS4*; DNA fragments of 100 ng were used.

flagellar biosynthesis and *fleQ* expression is closely associated with the flagellum-mediated motility (6, 12), we tested the effect of deleting or overexpressing *osaR* on swimming motility. As shown in Fig. 2C, deleting *osaR* did not influence swimming, while overexpressing *osaR* resulted in a significant loss of swimming ability. These results indicated that OsaR was a repressor of *fleQ*, but it remained unknown that why the absence of *osaR* has no effect on *fleQ* transcription.

OsaR binding site in *fleQ* promoter is a dual LTTR box that covers the −10 region and the transcription sites. To investigate the detailed mechanism by which OsaR represses *fleQ*, we further conducted gel shift assays and DNase I footprinting assay to identify the OsaR binding site in the intergenic region upstream of *fleQ*. DNA fragments that represent different parts of the whole intergenic region were amplified and tested for their affinity to OsaR (Fig. 3A). As shown in Fig. 3A and B, fragments F1, F2, and F6 were able to bind, while F3, F4, and F5 were unable to bind to OsaR, indicating that the overlapping region of F2 and F6, which was named F7, is the shortest fragment containing the OsaR binding site. In fact, a strong and clear motility shift was detected for F7

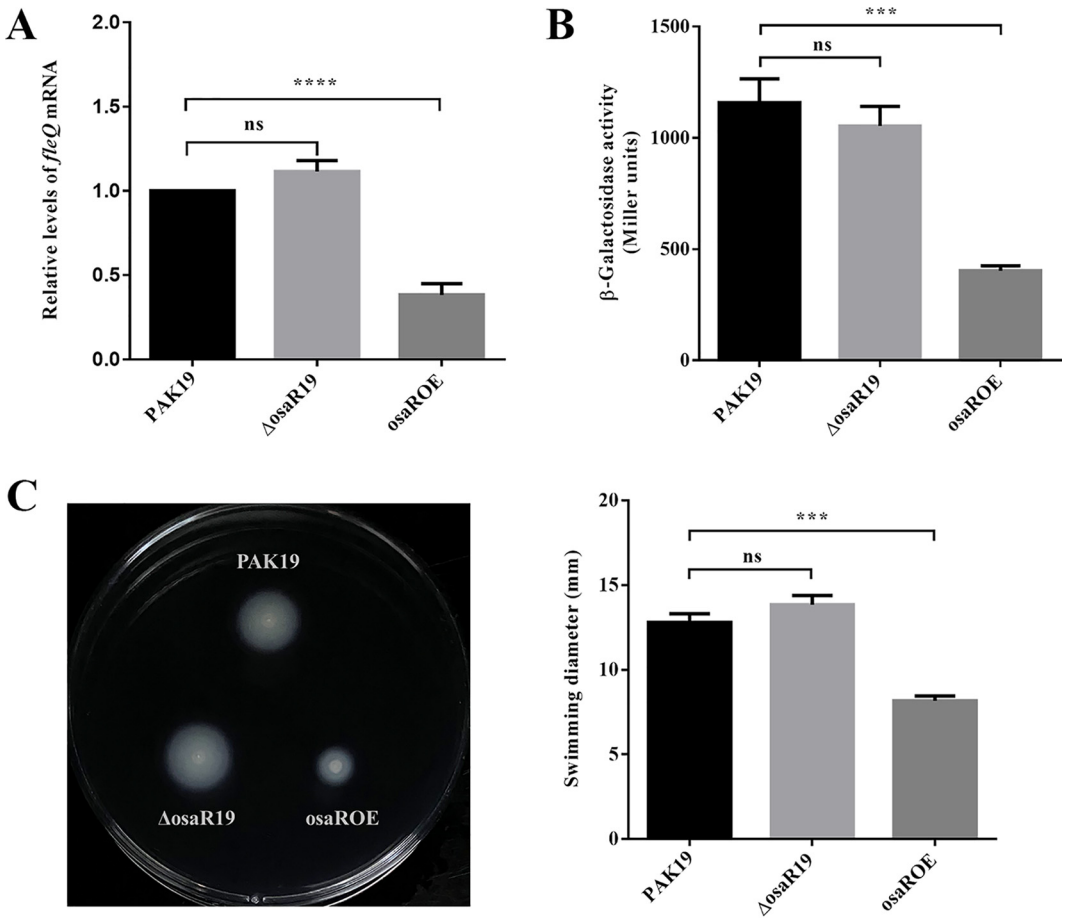

FIG 2 *osaR* overexpression downregulates *fleQ* and swimming motility. (A) The effect of OsaR on the transcriptional level of *fleQ*. The mRNA levels of *fleQ* were determined by qRT-PCR. (B) The effect of OsaR on the promoter activity of *fleQ*. WT strain (PAK19), *osaR* mutant (ΔosaR19), and *osaR* overexpressing strain (osaROE) were transformed with the reporter plasmid P_{fleQ}-*lacZ*, followed by β-galactosidase assays when cultured to an OD_{600} of ~0.6. (C) The effect of OsaR on swimming motility. Motility was detected on M63 glucose/CAA plates containing carbenicillin (left), and the diameters of the swimming zone were measured from three independent replicates (right). ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by Student's *t* test. Error bars represent standard deviations.

incubated with OsaR, whereas no shift was observed for a further shortened fragment, F8 (Fig. 3B). To determine the exact binding site, a 93-bp DNA fragment (F9) relative to the first base of the *fleQ*FP-F-FAM primer was used for DNase I footprinting assays. Based on F7, F9 was an extension that covered the −35 box for ensuring that the whole promoter was tested (Fig. 3A). The binding of OsaR to F9 was also verified (Fig. 3B). As shown in Fig. 3C, the fluorescence signal of fragments in lengths of 41 to 75 bp were reduced by addition of OsaR protein. It indicated that a region of approximately 35 bp in length was protected from DNase I digestion by OsaR, and the protection got stronger when the amount of OsaR was increasing, which implied this region was the OsaR binding site in the *fleQ* promoter. Based on the sequence of F9, the nucleotide sequence of the OsaR binding site was found to be 5′-ATAAAATTGACTAATCGTTCACATTTGACTTAACT-3′.

The generally accepted LTTR box consists of the sequence T-N_{11}-A, but this sequence can vary in both base pair composition and length in the reverse complementary region (21, 23). As indicated in Fig. 4A, the OsaR binding site in the *fleQ* promoter region is located from position −13 to +22, counting from the transcription start site, t2, that was previously reported (12). Examining the base sequence of this region, we found that two typical LTTR boxes, AT-N_{11}-AT (Box1) and A-N_{11}-T (Box2), were presented and distributed on either side of this region (Fig. 4A). We assumed that these two LTTR boxes were the core of the OsaR binding site. Based on the base sequence of fragment F7 (Fig. 3A), complementary

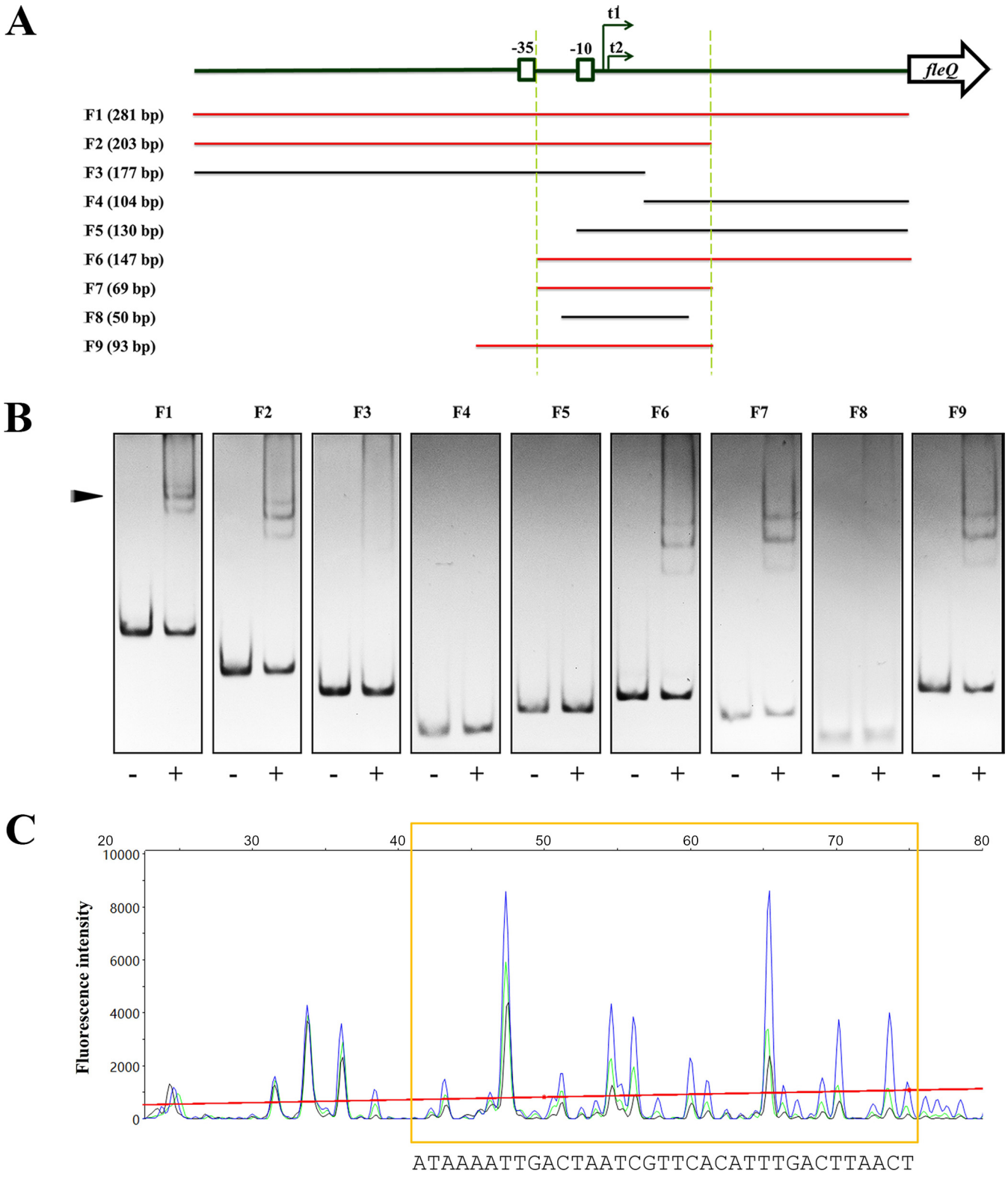

FIG 3 Identification of the exact binding sites of OsaR in the *fleQ* promoter. (A) Schematic diagram of the fragments used for gel shift assays and DNase I footprinting assays. The *fleQ* gene is represented by an open arrow. The −35 and −10 boxes of the *fleQ* promoter are boxed. The two transcription start sites, t1 and t2, are indicated by two arrows. The designation and length of each of the fragments, F1 to F9, are shown on the left; red and black colors indicate shift or no shift, respectively. The overlaps of F2 and F6 are defined between the two dashed lines. (B) Gel shift assays of OsaR and DNA fragments F1 to F9. The protein-DNA complex is indicated by an arrowhead. DNA fragments of 100 ng were used and incubated with (+) or without (−) OsaR at a concentration of 40 nM. All fragments were assayed on 5% polyacrylamide gels. F1 to F3, F4 to F6, and F7 to F9 were assayed on independent gels by electrophoresis for 1 h 50 min, 1 h 35 min, and 1 h 20 min, respectively. (C) A DNase I footprinting assay revealed the OsaR binding site. 5′-FAM-labeled

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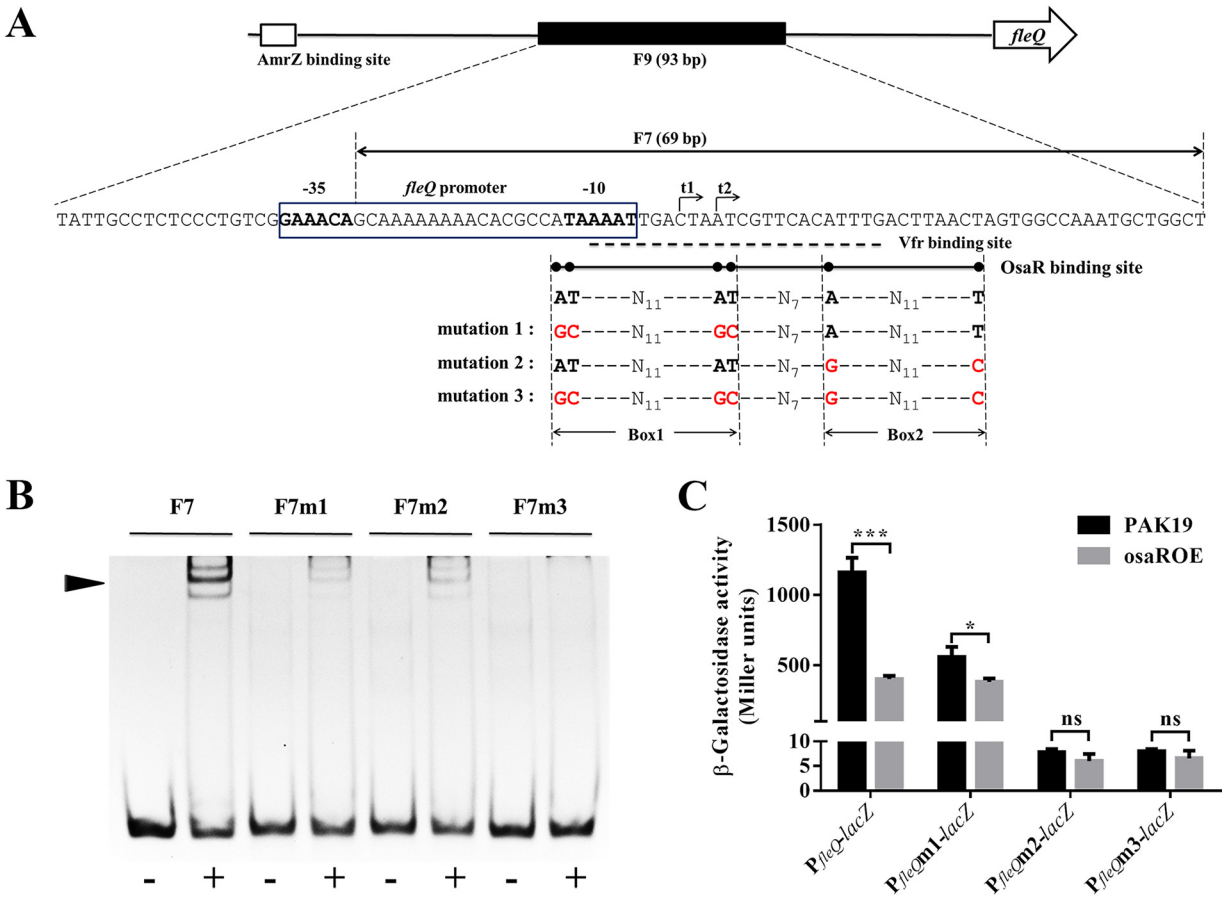

FIG 4 Characteristics of the OsaR binding site and their implications. (A) Schematic representation of the *fleQ* promoter region. The *fleQ* gene is represented by an open arrow. In the upstream intergenic region, the putative AmrZ binding site is represented by an open box, the F9 fragment used in the DNase I footprinting assay is represented by a solid box, and the base sequence matched to it is presented below. The F7 fragment is indicated by a double arrow. The *fleQ* promoter is enclosed in a box, and the −35 and −10 regions are boldfaced. The transcription start sites, t1 and t2, are indicated by arrows. The Vfr binding site is underlined with a dashed line. The OsaR binding site is underlined with a full line, in which the two pairs of the A-T palindromic bases are indicated by six dots. The sequence features of the OsaR binding site and the three types of base mutations are shown below; mutated bases are marked in red. (B) The binding affinity of OsaR for the F7 DNA fragment and its three different base mutants. F7m1, F7m2, and F7m3 correspond to the three base mutations in panel A. DNA fragments of 100 ng were used and incubated with (+) or without (−) OsaR at a concentration of 40 nM, followed by a 1.5-h electrophoresis on a 8% polyacrylamide gel. (C) The effect of key mutations in OsaR binding site on the promoter activity of *fleQ*. P_{fleQ}m1, P_{fleQ}m2, and P_{fleQ}m3 correspond to the three base mutations indicated in panel A. β-Galactosidase assays were conducted in WT strain and *osaR* overexpressing strain. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$; determined by Student's *t* test. Error bars represent standard deviations.

oligonucleotides were synthesized (Genewiz) with base mutations corresponding to mutation 1, mutation 2, and mutation 3 in Fig. 4A. Fragments F7m1, F7m2, and F7m3 were then generated by annealing the corresponding complementary oligonucleotides (shown in Table 2), thereby mutating the first, second, and both pairs of A-T palindromic bases of the OsaR binding site in F7, respectively. The binding affinity of OsaR to F7 and its base mutants was tested by gel shift assay, as shown in Fig. 4B. We also quantitated the percentage of each fragment that was shifted by using ImageJ software to measure the gray values. F7m1 and F7m2 both exhibited an obviously attenuated shift compared with the distinct shift of F7, while barely any shift was detected for F7m3 (Fig. 4B; see also Fig. S1 in the supplemental material). This result supports the specificity of the OsaR binding site. More importantly, it demonstrated that both of the A-T palindromic base pairs in Box1 and Box2 are vital for

FIG 3 Legend (Continued)
DNA fragment F9 (500 ng) was incubated with 80 nM (black), 40 nM (green), or 0 nM OsaR (blue) and then submitted to a 1-min DNase I treatment (0.5 U) and analyzed by capillary electrophoresis. The fluorescence intensity (arbitrary units, ordinate) is plotted against the sequence length (bases, abscissa) of the fragment. Peaks are superimposed, and the differentiated region of the three electropherograms is marked by a yellow box.

OsaR DNA recognition and affinity, which are weakened or even eliminated when they are altered. To further verify the importance of these two A-T pairs, we performed reporter assays with the three types of mutations indicated in Fig. 4A. As shown in Fig. 4C, mutation 1 lowered the promoter activity for PAK19, because two bases in the −10 box of the promoter were altered, but not for osaROE, due to the derepression caused by affecting the binding of OsaR. Overexpression of *osaR* decreased the activity by more than half (~750 Miller units reduced) and only about 1/3 (~180 Miller units reduced) of WT *fleQ* promoter and mutated *fleQ* promoter, respectively. Unexpectedly and intriguingly, mutation 2 and mutation 3 led to extremely low expression of *lacZ* for both strains. We then examined the transcription of *lacZ* by RT-qPCR and found that the mRNA of *lacZ* did not plunge to such a low level (Fig. S2). In addition, as shown in Fig. S2, *lacZ* transcription was even increased by ~2-fold when the promoter was mutated in the *osaR* overexpressing strain (osaROE) but still lower than that in the WT strain (PAK19), which supports the result that mutation 1 detracts but does not abolish OsaR binding. We speculate that the common mutated bases of mutation 2 and mutation 3, that is, the A-T pair of Box2, plays a crucial role in the translation of the gene downstream of the *fleQ* promoter. Further investigations are ongoing now.

Therefore, we can conclude that the OsaR binding site in the *fleQ* promoter is a characteristic sequence, AT-N_{11}-AT-N_7-A-N_{11}-T. It is highly similar to the consensus binding sequence of another well-studied LTTR, OxyR (24, 25) (Fig. S3A). We have also identified it in the promoter region of other genes that are regulated or putatively regulated by OsaR, such as *PA0057* (Fig. S3B) (20).

OsaR and Vfr function as redundant repressors of *fleQ*. It was previously reported that the virulence factor regulator Vfr was also able to repress the transcription of *fleQ*; overexpression of Vfr resulted in a reduction in *fleQ* promoter activity and motility, whereas no effect was shown with *vfr* deletion (12). Our study identified that OsaR represses the transcription of *fleQ* and binds to a promoter region overlapping the Vfr binding site (Fig. 2A and B and 4A). Two transcription factors binding to the same position implies a coregulatory mechanism. We first confirmed the function of Vfr. As shown in Fig. S4, deleting *vfr* did not affect *fleQ* mRNA level and promoter activity or bacterial motility; however, they were prominently reduced in the *vfr*-overexpressing strain.

Therefore, OsaR and Vfr display an identical role in regulating *fleQ*. In particular, either of the two repressors being absent has no significant effect on *fleQ* transcription, suggesting that their function is redundant. To validate this assumption, we knocked out both *osaR* and *vfr* in PAK and then tested the influence on *fleQ* expression. As shown in Fig. 5, the double-knockout strain exhibited upregulated mRNA levels and promoter activity of *fleQ* and increased swimming ability. In addition, these phenotypes could be restored with the expression of either *osaR* or *vfr* alone (Fig. 5). It is worth noting that the level of *fleQ* transcription in the ΔosaRΔvfr strain was only approximately 2-fold upregulated compared with the wild-type strain (Fig. 5). AmrZ is likely responsible for this. In the absence of AmrZ, *fleQ* promoter activity and mRNA levels were upregulated by approximately 4-fold and 8-fold, respectively (15, 16). This suggests that AmrZ is a more effective repressor of *fleQ* than OsaR and/or Vfr.

In conclusion, our results demonstrated that OsaR and Vfr separately bind to the same position covering the entire (OsaR) or partial (Vfr) −10 box and the two transcription start sites in the *fleQ* promoter. The redundant repression would be attenuated only when the two repressors were simultaneously removed, because either one of them could retain the repression when the other one was absent.

DISCUSSION

The regulator gene *fleQ* is vital for the expression of almost all known flagellar genes in *P. aeruginosa* (6–8). Mechanisms that inactivate or downregulate *fleQ* are sufficient to inhibit flagellum biosynthesis as well as flagellum-mediated motility (7, 12, 26). Overexpression of *fleQ* in a nonmotile *P. aeruginosa* strain increased flagellar numbers and restored flagellum-mediated motility (26). It was reported that *fleQ* was downregulated by overexpression of the virulence factor regulator Vfr (12); however, it was perplexing that knocking out *vfr* had no effect on *fleQ* (12). In this study, we unraveled this conundrum and provided updated

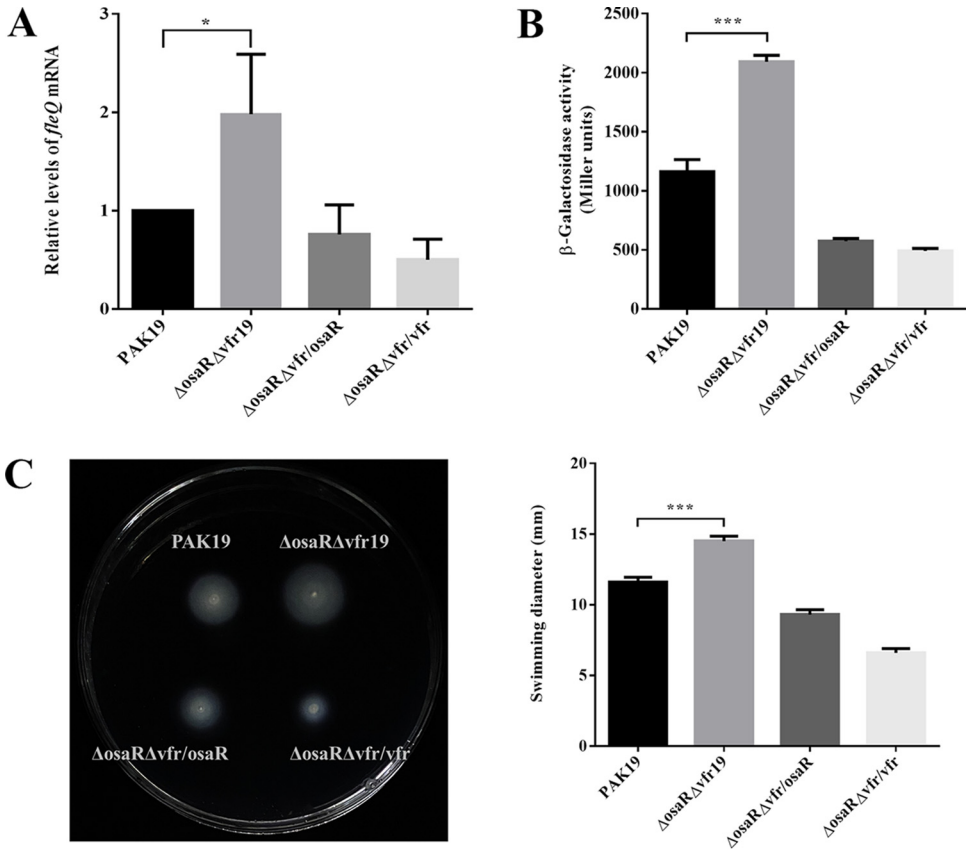

FIG 5 *fleQ* and swimming motility are upregulated when *osaR* and *vfr* are both deleted. (A) The effect on *fleQ* transcription by deleting *osaR* and *vfr* simultaneously. The mRNA levels were determined by RT-qPCR. (B) The effect on the promoter activity of *fleQ* by deleting both *osaR* and *vfr*. Strains were transformed with the reporter plasmid P_{fleQ}-*lacZ*, followed by β-galactosidase assays when cultured to an OD_{600} of ~0.6. (C) The effect on swimming motility by deleting both *osaR* and *vfr*. Motility was detected and quantitated by three independent replicates. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$; determined by Student's *t* test. Error bars represent standard deviations.

demonstrations about the upstream regulation of *fleQ*. We identified a protein-DNA interaction between the LysR-type transcriptional regulator OsaR and the promoter of *fleQ*. Further experimental results revealed that OsaR can bind to the −10 region and retain the transcriptional repression when Vfr is absent and vice versa.

Many bacterial promoters are controlled by multiple transcription factors with positive or negative interplay, which illustrates the complexity, diversity, and versatility of bacterial gene regulation mechanisms (27–30). The binding sites of competitive repressors partially overlap the control region, and a substantial boost of expression of the controlled gene will only be generated by removing the repressors simultaneously (27, 28, 30). The regulatory pattern of OsaR and Vfr repressing *fleQ* identified by our study is compatible with the canonical mode of competitive repression, except *fleQ* only displays modest upregulation in the ΔosaRΔvfr double mutant. We suppose that another repressor, AmrZ, could still repress *fleQ* to a certain extent when OsaR and Vfr are both absent. Depending on the alternative sigma factor AlgT (31), AmrZ directly interacts with the *fleQ* promoter and effectively suppresses *fleQ* transcription (15, 26). Dissimilar to the OsaR/Vfr binding sites, however, the putative AmrZ binding site is located further upstream of the −35 region (Fig. 4A) according to previous ChIP-seq analysis (16). Although further investigation is needed for identifying the specific relationship among the regulation by the three repressors, our discovery indicates the potential of distinct manners in which OsaR/Vfr and AmrZ repress *fleQ* independently.

Our previous study found that OsaR was involved in bacterial tolerance to antibiotics and oxidative stress (20). OsaR protein undergoes a change in aggregation state

between its oxidative state and reductive state and shows different DNA affinity under different redox conditions (20). Therefore, oxidative stresses, including those generated by antibiotics, might be the signaling factor that controls OsaR activity, which is similar to another LTTR, OxyR, that senses reactive oxygen species (ROS) and regulates target genes in a redox-dependent manner (24, 32). Hence, our study might provide critical clues for understanding the motility changes of *P. aeruginosa* mutants that display altered susceptibility to antibiotics or oxidative stress (33–35).

Members of the LTTR family have a highly conserved amino acid composition and secondary structure in their DNA-binding domain as well as a characteristic binding sequence, called the LTTR box, which consists of the sequence T-N_{11}-A, which can vary in both base pair composition and length (21, 23). The OxyR binding sites in antioxidant genes exhibit typical characteristics of the LTTR box (24, 25), including two LTTR boxes with a spacer composed of 7/8 random bases (see Fig. S3A in the supplemental material). In this report, the OsaR binding site we identified in *fleQ* was AT-N_{11}-AT-N_7-A-N_{11}-T (Fig. 4A), which is highly similar to the OxyR binding sites. In addition, our previous work indicated that an approximately 40-bp section of DNA within the intergenic region of *PA0056-PA0057* was protected from DNase I digestion by the OsaR protein (20). The features of a dual LTTR box were found in the base sequence, which were the same as those of the *fleQ*-bound site (Fig. S3B). The available evidence suggests that AT-N_{11}-AT-N_7-A-N_{11}-T, or its variant, is the OsaR consensus sequence, and, based on this, further investigation of the global function of OsaR could be conducted. Moreover, because OsaR and OxyR both transform between oxidation and reduction states (20, 24, 32) and share a binding sequence with high similarity, the potential for cross talk between these two LTTRs appears to have research prospects. Intriguingly, in a study by Panmanee et al. (36), bioinformatics analysis was performed to screen OxyR binding sites in *P. aeruginosa* based on similarity to the well-characterized *Escherichia coli* OxyR-regulated promoter sequences (ATAG-N_7-CTAT-N_7-ATAG-N_7-CTAT), and *fleQ* was identified as one of the OxyR-dependent gene candidates. There was no further evidence supporting the OxyR-*fleQ* interaction in the aforementioned study; however, our study demonstrated that *fleQ* is under the regulation of OsaR.

Classical LTTR regulation has been described as transcriptional activation and negative autoregulation, and there are relatively fewer reports of LTTRs acting as transcriptional repressors that positively autoregulate (21). LrhA was first identified in *E. coli* as a LysR homolog (37), and, with its homologs HexA and PecT in *Erwinia* spp. (38), subsequent studies revealed that these three LTTRs positively autoregulate themselves and negatively regulate the expression of genes required for flagellation, motility, and chemotaxis (21, 37–39). In *E. coli*, the *flhDC* operon encodes the master regulator in the hierarchical regulation of flagellar biogenesis (40); it is activated by the cyclic AMP (cAMP) receptor protein CRP (41) and negatively regulated by LrhA (39). In *P. aeruginosa*, we previously identified that *osaR* expression is positively autoregulated by OsaR (20); here, we demonstrated that the top-level regulator in the flagellar biogenesis hierarchy, FleQ, is repressed redundantly by the LTTR OsaR and the cAMP receptor protein Vfr, which is actually a homolog of *E. coli* CRP (42). We speculate that coregulation mechanisms of the flagellation-related master regulator involving a positively autoregulated LTTR and a cAMP receptor protein is conserved in multiple bacterial species.

In addition to *P. aeruginosa*, FleQ appears to be at the top level of the hierarchical regulation of flagellar biogenesis in all of the examined pseudomonads, such as *P. putida* and *P. fluorescens* (43). FleQ homologs that regulate flagellar biosynthesis are also widely distributed in other bacterial species, including FlrA in *Vibrio cholerae* (44), FlaK in *Vibrio parahaemolyticus* (45), and FlgR in *Helicobacter pylori* (46). Therefore, any further elucidation of the upstream regulatory mechanisms of *fleQ* will be remarkably significant to the study of all of the related flagellated bacteria, especially because, to the best of our knowledge, OsaR and its homologs in other species have not been reported yet.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* and *Escherichia coli* strains were routinely grown on LB medium at 37°C. Additionally, M63 glucose/CAA medium (M63 minimal medium supplemented with 0.2% glucose, 1 mM $MgSO_4$, and 0.5% Casamino Acids) was used to detect motility (47–49).

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	CWBIO
BL21	F$^-$ *ompT hsdS*(r_{BB}^- m_B^-) *gal dcm* (DE3)	CWBIO
S17-1	RP4-2 Tc::Mu Km::Tn*7* Tp^r Sm^r Pro Res$^-$ Mod$^+$	R. Ramphal
S17/pEX18Tc-Δ*vfr*	S17-1 harboring the pEX18Tc-Δ*vfr* vector; Tc^r	58
BL21/pETMAL-*osaR*	BL21 harboring the pETMAL-*osaR* vector; Kan^r	20
P. aeruginosa		
PAK	Wild type	David Bradley
PAK19	PAK harboring the pUCP19 vector; Cb^r	This study
ΔosaR	PAK with *osaR* gene deleted	20
ΔosaR19	ΔosaR harboring the pUCP19 vector; Cb^r	This study
Δvfr	PAK with *vfr* gene deleted	58
Δvfr19	Δvfr harboring the pUCP19 vector; Cb^r	This study
ΔosaRΔvfr	PAK with both *osaR* and *vfr* genes deleted	This study
ΔosaRΔvfr19	ΔosaRΔvfr harboring the pUCP19 vector; Cb^r	This study
osaROE	PAK harboring the pUCP19-*osaR* vector for overexpression of *osaR*; Cb^r	20
vfrOE	PAK harboring the pKF912 vector for overexpression of *vfr*; Cb^r	This study
ΔosaRΔvfr/osaR	ΔosaRΔvfr mutant harboring the pUCP19-*osaR* vector for complement of *osaR*; Cb^r	This study
ΔosaRΔvfr/vfr	ΔosaRΔvfr mutant harboring the pKF912 vector for complement of *vfr*; Cb^r	This study
Plasmids		
pEX18Tc-Δ*vfr*	*vfr* gene deletion on pEX18Tc; Tc^r	58
pETMAL-*osaR*	pETMALc-H vector carrying the malE-OsaR fusion; Kan^r	20
pUCP19	Multicopy *E. coli-P. aeruginosa* shuttle vector; Ap^r/Cb^r	59
pUCP19-*osaR*	pUCP19 carrying the intact *osaR* gene; Ap^r/Cb^r	20
pKF912	pUCP19 carrying *vfr* as a 1.2-kb XhoI fragment; Ap^r/Cb^r	42
pDN19lacΩ	Broad-host-range plasmid containing a promoterless *lacZ* gene; Tc^r	60
P_{fleQ}-*lacZ*	pDN19lacΩ carrying the promoter region of *fleQ* in the EcoRI/BamHI sites; Tc^r	This study
P_{fleQ}m1-*lacZ*	P_{fleQ}-*lacZ* with the first A-T pair of the OsaR binding site mutated; Tc^r	This study
P_{fleQ}m2-*lacZ*	P_{fleQ}-*lacZ* with the second A-T pair of the OsaR binding site mutated; Tc^r	This study
P_{fleQ}m3-*lacZ*	P_{fleQ}-*lacZ* with the two A-T pairs of the OsaR binding site mutated; Tc^r	This study

Antibiotics were added at the following concentrations: 50 μg/ml tetracycline or 150 μg/ml carbenicillin for *P. aeruginosa*; 10 μg/ml tetracycline, 25 μg/ml kanamycin, or 50 μg/ml carbenicillin for *E. coli*.

The bacterial strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table 2. The plasmids were constructed based on standard DNA manipulations with the *E. coli* DH5α strain and confirmed by sequencing (Genewiz), after which they were transformed into *P. aeruginosa* strains by electroporation (50). The construction of the ΔosaRΔvfr mutant was based on the ΔosaR mutant and the S17/pEX18Tc-Δ*vfr* strain; the conjugation and the selection of double-crossover mutants were conducted according to manipulations previously reported (51).

Protein expression and purification. Expression and purification of OsaR were conducted as previously described (52), with slight modifications. Briefly, the pETMALc-H vector was used to construct an MBP-OsaR fusion expression plasmid to enhance the solubility of the expressed protein. The BL21/pETMAL-*osaR* strain was cultured in LB medium supplemented with 0.2% glucose overnight, followed by a 100-fold dilution into fresh medium. The culture was grown at 37°C until the optical density at 600 nm (OD_{600}) reached 0.6, and then 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction for another 2 h at 28°C. The cells then were harvested and suspended in PB buffer (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 7.4) supplemented with 500 mM NaCl and 1 mM EDTA. The OsaR protein was acquired and purified based on the pMAL protein fusion and purification system manual (NEB E8200), in which amylose resin (NEB) was used for affinity chromatography and thrombin (Macklin) was used for the cleavage of the fusion protein.

Gel shift assay. To assess the ability of OsaR to bind to the promoter of *fleQ* in gel shift assays, various desired fragments of the intergenic region upstream of the translation start site were amplified by PCR using different pairs of primers (primers shown in Table 2). Site-directed mutagenesis fragments were obtained by annealing complementary oligonucleotides (base sequences shown in Table 2). All of the DNA fragments used for the gel shift assay were purified with the MiniBEST agarose gel DNA extraction kit (TaKaRa). The binding and shift assays were performed as previously published (53, 54). Purified protein and DNA fragments were mixed in a 25-μl mixture containing the gel-shift binding buffer (20 mM Tris-HCl, 50 mM KCl, 50 mM $MgCl_2$, 10% glycerol, pH 7.5) and subsequently incubated at 37°C for 30 min. The samples were resolved on a 5% or 8% polyacrylamide native gel in Tris-borate-EDTA buffer (0.044 M Tris, 0.044 M boric acid, 0.001 M EDTA, pH 8.0) on ice. Gels were soaked in 0.5 μg/ml ethidium bromide (EB), and then the DNA was visualized under UV light and imaged in a MultiImage light cabinet filter position molecular imager (Alpha Innotech Corporation).

DNase I footprinting assay. DNase I footprint analysis was performed using a nonradiochemical capillary electrophoresis method as previously described (55, 56). A 93-bp DNA fragment was generated

TABLE 2 Primers used in this study

Oligonucleotide	Sequence (5′–3′)[b]	Purpose
PfleQ-F[a]	CCGGAATTCGTTTTTCATGGCTTTGTGCCG	Construction of P_{fleQ}-*lacZ*
PfleQ-R	CGCGGATCCTTTGATCAGCTGCCTTGCATC	Construction of P_{fleQ}-*lacZ*
pKF912-F	CCCAAGCTTGAAGGCTTCGCAGCTCTC	Construction of pKF912
pKF912-R	GGAATTCCTGAAGCGCCTGTTCTTCC	Construction of pKF912
Δ*vfr*-F	CGATGATGTCGACAACATCG	Identification of *vfr* deletion
Δ*vfr*-R	TCAAGAGCCAGACCCTGATG	Identification of *vfr* deletion
*fleS*1-F	GGCATGAGCCGGCGTGAC	Amplification of *fleS*1
*fleS*1-R	CGTATCGGCTGGCTGCTC	Amplification of *fleS*1
*fleS*2-F	ATGCAACCAGCCCTCAACGC	Amplification of *fleS*2
*fleS*2-R	TGACGATGACGCCGCCGG	Amplification of *fleS*2
*fleS*3-F	GCGGAAAAGGAGCGCCTGG	Amplification of *fleS*3
*fleS*3-R	GTTGAGCAGGATCAGTTGCC	Amplification of *fleS*3
*fleS*4-F	AGCCGTTGGTGGGCATGC	Amplification of *fleS*4
*fleS*4-R	CGGGCGAACACCAGCATG	Amplification of *fleS*4
*fleQ*F123-F	GTTTTTCATGGCTTTGTGCC	Amplification of F1, F2, F3
*fleQ*F1456-R	TTTGATCAGCTGCCTTGCATC	Amplification of F1, F4, F5, F6
*fleQ*F2-R	AGCCAGCATTTGGCCACTA	
*fleQ*F3-R	CAAATGTGAACGATTAGTCA	Amplification of F2
*fleQ*F4-F	ACTTAACTAGTGGCCAAATGC	Amplification of F3
*fleQ*F5-F	TAAAATTGACTAATCGTTCAC	Amplification of F4
*fleQ*F6-F	GCAAAAAAAACACGCCATAA	Amplification of F5
*fleQ*F7-F	GCAAAAAAAACACGCCATAAAATTGACTAATCGTTCACATTTGACTTAACTAGTGGCCAAATGCTGGCT	Amplification of F6
*fleQ*F7-R	AGCCAGCATTTGGCCACTAGTTAAGTCAAATGTGAACGATTAGTCAATTTTATGGCGTGTTTTTTTTGC	Annealing of F7
*fleQ*F8-F	ACACGCCATAAAATTGACTAATCGTTCACATTTGACTTAACTAGTGGCCA	Annealing of F7
*fleQ*F8-R	TGGCCACTAGTTAAGTCAAATGTGAACGATTAGTCAATTTTATGGCGTGT	Annealing of F8
*fleQ*F7m1-F	GCAAAAAAAACACGCCGCAAAATTGACTAGCCGTTCACATTTGACTTAACTAGTGGCCAAATGCTGGCT	Annealing of F8
*fleQ*F7m1-R	AGCCAGCATTTGGCCACTAGTTAAGTCAAATGTGAACGGCTAGTCAATTTTGCGGCGTGTTTTTTTTGC	Annealing of F7m1
*fleQ*F7m2-F	GCAAAAAAAACACGCCATAAAATTGACTAATCGTTCACGTTTGACTTAACCAGTGGCCAAATGCTGGCT	Annealing of F7m1
*fleQ*F7m2-R	AGCCAGCATTTGGCCACTGGTTAAGTCAAACGTGAACGATTAGTCAATTTTATGGCGTGTTTTTTTTGC	Annealing of F7m2
*fleQ*F7m3-F	GCAAAAAAAACACGCCGCAAAATTGACTAGCCGTTCACGTTTGACTTAACCAGTGGCCAAATGCTGGCT	Annealing of F7m3
*fleQ*F7m3-R	AGCCAGCATTTGGCCACTGGTTAAGTCAAA	Annealing of F7m3
*fleQ*FP-F-FAM	CGTGAACGGCTAGTCAATTTTGCGGCGTGTTTTTTTTGC	DNase I footprinting assay
*fleQ*FP-R	TTTATTGCCTCTCCCTGTCG	DNase I footprinting assay
fleQ-qRT-F	AGCCAGCATTTGGCCACTA	qRT-PCR
fleQ-qRT-R	ACTACCGCCTCAACGTATTCC	qRT-PCR
rpsL-qRT-F	CGCTTCTCATGCTCCATCC	qRT-PCR
rpsL-qRT-R	ACGTGCCTGCGCTGCAAAACCCCGAGGTGTCCAGCGAACC	qRT-PCR

[a]F, forward; R, reverse; FAM, 6-carboxyfluorescein phosphoramidate labeled at the 5′ end.
[b]The underlines are the sites of restriction enzymes.

by PCR using a 6-FAM (6-carboxyfluorescein phosphoramidate) primer, *fleQ*FP-F-FAM, labeled at the 5′ end and paired with the primer *fleQ*FP-R (primers are shown in Table 2). The labeled DNA fragment was purified with the MiniBEST agarose gel DNA extraction kit (TaKaRa) and then mixed with purified OsaR protein in 100-μl reaction mixtures containing the same binding buffer as that used in the gel shift assays. After incubation for 30 min at room temperature, 0.5 U of DNase I (Invitrogen) was added for 1 min of further incubation. The samples were then placed at 70°C in a water bath for 10 min to end the reaction, followed by purification utilizing the UNIQ-10 spin column Oligo DNA purification kit (Sangon Biotech). Fragments were analyzed by capillary electrophoresis, and the sizes were determined using ABI Peak Scanner software v2.0.

RNA extraction and RT-qPCR. Overnight culture of *P. aeruginosa* strains was diluted 1/100 into fresh LB medium, followed by subculturing until the OD_{600} reached 1.0. The bacterial cells were subsequently harvested, and total RNA was isolated utilizing the SPARKeasy bacterial RNA kit (SparkJade). cDNA was synthesized with the PrimeScript RT reagent kit (TaKaRa), including a genomic DNA erasing step followed by reverse transcription-PCR with random primers. Real-time PCR was performed using the cDNA as the template, which was mixed with the indicated primers (shown in Table 2) and qPCR SYBR green master mix (Yeasen). The gene *rpsL* encoding the 30S ribosomal protein was used as internal control.

P_{fleQ}-*lacZ* reporter assay. P_{fleQ}, which represents the intergenic region upstream of *fleQ* or its three variants, P_{fleQ}m1, P_{fleQ}m2 and P_{fleQ}m3, were inserted upstream of the promoter-less *lacZ* gene in the pDN19lacΩ vector, generating P_{fleQ}-*lacZ*, P_{fleQ}m1-*lacZ*, P_{fleQ}m2-*lacZ*, and P_{fleQ}m3-*lacZ*, respectively. P_{fleQ} was amplified by PCR; P_{fleQ}m1, P_{fleQ}m2, and P_{fleQ}m3 were synthesized by Genewiz. The transcriptional fusion plasmids were transformed into specific strains for conducting β-galactosidase assays, and the β-galactosidase assay was carried out as previously described (57).

Motility assay. Motility was assessed as previously described; M63 glucose/CAA medium with 0.3% agar was used for the swimming motility assay (47–49). Fresh bacterial colonies were inoculated onto the surface of the plate with sterile toothpicks, followed by 12 to 16 h of incubation at 30°C.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

This work was supported by the Sino-Swiss Scientific and Technological Cooperation Project, supported by the Ministry of Science and Technology of China (no. 2015DFG32140) and the National Natural Science Foundation of China (no. 31770102).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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